Purification of Plant Cell Walls: Isoelectric Focusing of $CaCl_2$ Extracted Enzymes

Summary

A preparative procedure for cell wall isolation and purification was developed. The purity of the isolated cell walls was judged biochemically by the lack of activity of cytoplasmic marker enzymes and morphologically by examination at both the light and electron microscope levels. The purified cell walls were extracted with various salt treatments and the molecular weight range of most of the extracted proteins was between 14 and 31 kDa. The salt extracted hydrolytic enzymes were basic in nature (pI > 7.0) compared to their cytosolic counterparts (pI < 7.0). Some enzymes were readily extracted from cell walls (β-glucosidase and β-NAcglucosaminidase) with high salt treatment while most of the α-mannosidase activity associated with purified cell walls could not be removed even with sequential high salt treatments.

Keywords: Isoelectric focusing; Isolation; Potato tuber cell walls; Purification; Salt extraction; Ultrastructure.

1. Introduction

Cell wall associated protein molecules such as extensin and potato tuber agglutinin, that may not have enzymatic functions, have a high isoelectric pH (pI) between 9.65 to 12.0 (COOPER *et al.* 1984, LEACH *et al.* 1982). In contrast, limited information is available on the pI values of cell wall associated enzymes (BORCHERT and DECEDUE 1978, HÖSEL *et al.* 1978). Although the cell wall enzymes are likely to be basic in nature, the evidence is not compelling (GREVE and ORDIN 1977).

In our previous report (NAGAHASHI *et al.* 1985), we developed an analytical cell wall purification procedure which was not satisfactory for obtaining large amounts of cell wall protein. This study involved the development of a preparative cell wall purification procedure in order to bulk extract protein to use for column isoelectric focusing (IEF) in a density gradient. Large amounts of very pure cell walls are necessary to study 1. how various enzymes are bound to the wall, 2. the molecular properties of these enzymes, and 3. the possible biological functions of these enzymes.

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2. Materials and Methods

2.1. Cell Disruption Procedure

Fresh potato tubers (cv. Kennebec) were obtained from Beltsville Agricultural Research Center and stored at 4 °C. Approximately 454 g of peeled tubers were processed through an ACME Supreme Juicerator (30 seconds) and the pulp was collected on the miracloth lined basket. The pulp was immediately suspended in 400 ml of homogenization medium (0.3 M sucrose, 0.1 M HEPES-MES pH 7.8, 5 mM β-mercaptoethanol and 2 mM $Na_2S_2O_5$) and homogenized in a Tissumizer for 60 seconds to generate clumps of tissue small enough to be extruded by a Parr nitrogen bomb. The homogenate was immediately filtered on 1 layer of cheesecloth and the trapped walls were washed twice with cold deionized water. The crude preparation was suspended in 700 ml with homogenization medium, transferred to the nitrogen bomb, and equilibrated at 1500 PSI for 10 minutes at 4 °C. To efficiently extrude the contents, the bomb was recharged several times and the pressure during extrusion was never allowed to go below 1000 PSI. The crude cell walls were trapped on cheesecloth, washed, and resuspended in 300 ml fresh homogenization medium before sonication (Fig. 1). Sonication was performed at 4 °C with a Sonicator Cell Disruptor Model-225 R (Heat Systems—Ultrasonics, Inc.) for 5 minutes at output control 7 with a 50% pulsed cycle. The yield of purified cell wall averaged 3.5 g dry weight/454 g of fresh weight potato tuber and was directly lowered by an inefficient extrusion of the homogenate from the bomb. Other plant tissues can be used in the Parr bomb however the initial tissue disruption step may have to be modified as indicated in Fig. 1.

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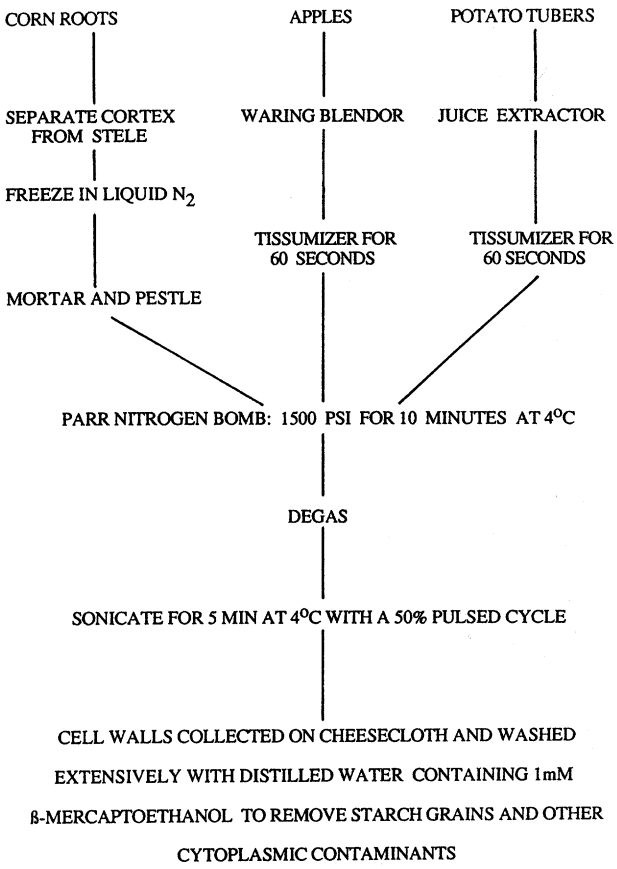

Fig. 1. Flow diagram of the potato tuber cell wall isolation procedure. The procedure can be modified for other tissue types by changing the initial tissue disruption step. This step was necessary to generate small pieces of intact tissue which could pass through the orifice of the Parr bomb. Cell disruption in the nitrogen bomb reduces the damage done by polyphenolic oxidases and is a rapid way to get nearly complete cell breakage. Sonication was necessary to break the few remaining intact cells and to jar loose the adhering cytoplasmic contaminants

2.2. Assays

Glycosidases were assayed with 5 mM paranitrophenyl (PNP)-glycosides at pH 4.5. Acid phosphatase activity was determined with paranitrophenyl-phosphate at pH 4.5. All of these assays were performed at 38 °C and terminated with 1.0 M Na_2CO_3 after 5 to 30 minutes incubation (NAGAHASHI *et al.* 1985). PNP released was measured at 405 nm and compared to a p-nitrophenol standard. Cytochrome c oxidase (CCO) and malate dehydrogenase (MDH) were assayed as described previously (NAGAHASHI *et al.* 1985, TING *et al.* 1975). Protein was estimated by a modified Lowry procedure (MARKWELL *et al.* 1978). To assay wall suspensions, the disposable tip of the automatic pipetor was cut back to increase the bore size. This was essential for pipeting homogeneous suspensions of cell walls. Cell walls were assayed for 10 to 30 minutes with 0.38 to 0.59 mg dry weight per assay. Immediately after termination of enzyme reactions, the tubes were centrifuged to pellet the cell walls and an aliquot of the clear supernatant was read in a Beckman Model 35 spectrophotometer.

2.3. Electrophoresis

Preparative IEF was performed in a 110 ml LKB 8100-1 column at 4 °C. The pH gradient (pH 3 to 10) contained a final ampholyte concentration of 2% and was stabilized in a linear glycerol gradient (0–52%). Gradients were focused for 20 hours at 1500 V, fractionated into 2 ml fractions, and pH was determined at 4 °C.
Proteins extracted from purified cell walls were also separated by SDS polyacrylamide (7.5%) gel electrophoresis. Gels were stained with Coomassie blue and scanned with an ISCO model 1310 gel scanner at 580 nm.

2.4. Extraction, Concentration and Dialysis of Cell-Wall Associated Proteins

Various salts (as indicated in the text) were used to solubilize ionically bound-cell-wall associated enzymes. Each extraction step was performed for 12–15 hours (3 changes/salt) in the presence of 1 mM 2-mercaptoethanol. After each step, cell walls were exhaustively washed in 1 mM 2-mercaptoethanol and aliquots were assayed directly for various hydrolytic enzymes. The salt extracts were concentrated under nitrogen pressure in an Amicon apparatus (PM 10 membrane). Concentrated extracts were placed in dialysis tubing (MW cutoff 3,500) and dialyzed overnight against 1 mM 2-mercaptoethanol.

2.5. Microscopy

Light microscopy was performed with an Olympus BHA microscope and camera. Electron microscopy was performed with a Zeiss EM 10 B electron microscope. Isolated cell wall fractions were fixed in 2.5% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.0). Fixed cell walls were collected on 0.2 micron filters, post-fixed with 1% OsO_4, dehydrated in acetone, embedded in Spurr's epoxy resin and thin sectioned.

3. Results and Discussion

3.1. Purification of Cell Walls

The Parr nitrogen bomb provided an ideal cell disruption step since the denaturation of proteins by the products of polyphenol oxidase was minimized by the N_2 atmosphere. Furthermore, the nitrogen bomb provided a rapid, low shear method for obtaining large quantities of large cell wall pieces. Sonication was essential to break the few remaining intact cells after application of the nitrogen bomb and to release the sticky fragments of cytoplasm (NAGAHASHI *et al.* 1985). The washing technique required the trapping of large pieces of cell wall on a sieve (cheesecloth in this case) so that cytoplasmic contaminants could be washed away. Centrifugation was avoided since washing by repelleting and resuspension was not an efficient way to remove contaminants from cell walls (NAGAHASHI *et al.* 1985, LI *et al.* 1983).
Light microscopy of intact potato tuber tissue showed that starch bodies would be a major contaminant of cell walls (micrograph not shown). This was confirmed by

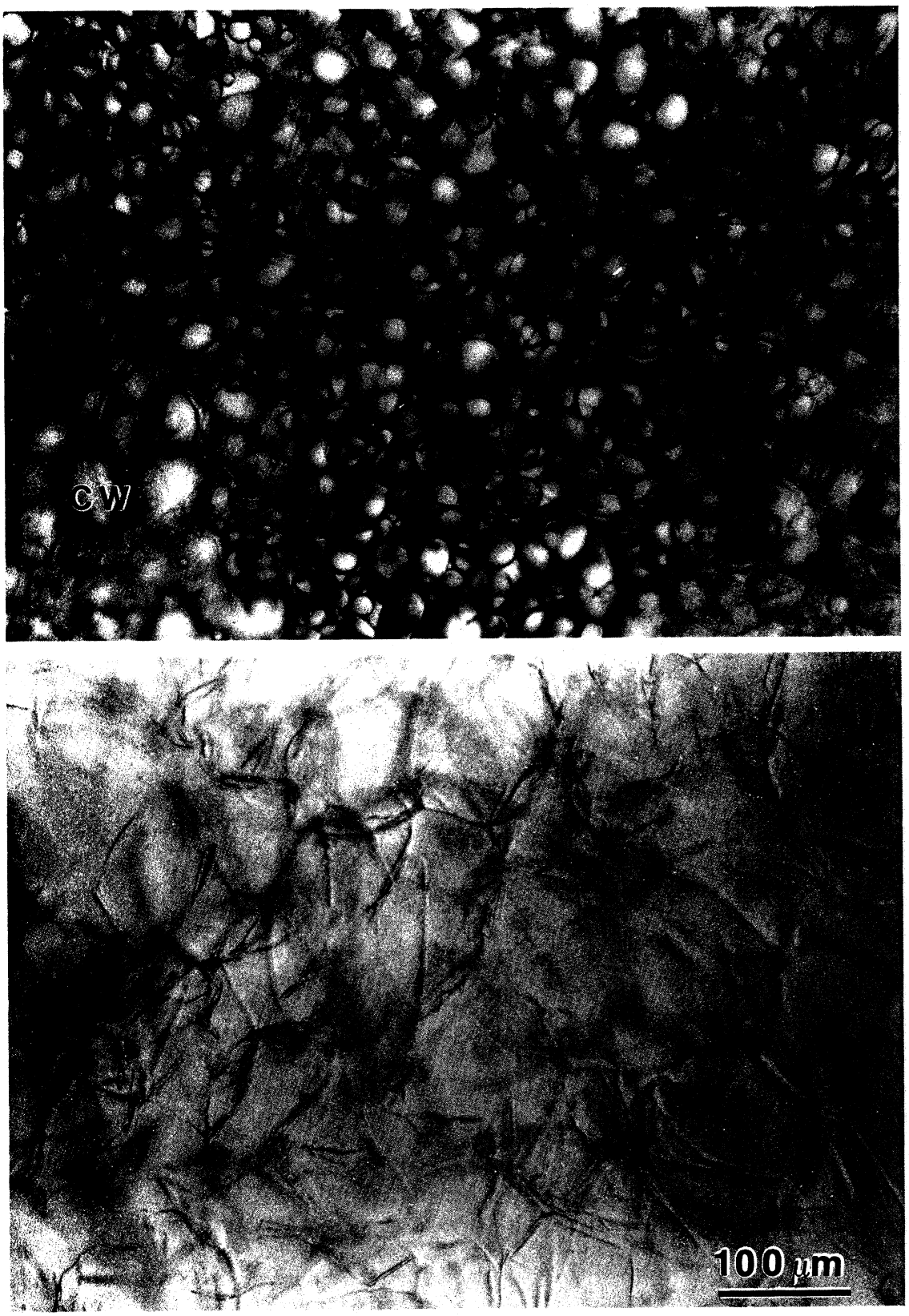

Fig. 2. Photomicrographs of crude walls and purified cell walls isolated from potato tubers. Wet mounts were stained with India ink to enhance the contrast between cell walls and starch grains. Top: Crude cell wall fraction. Bottom: Purified cell wall fraction. Both at same magnification

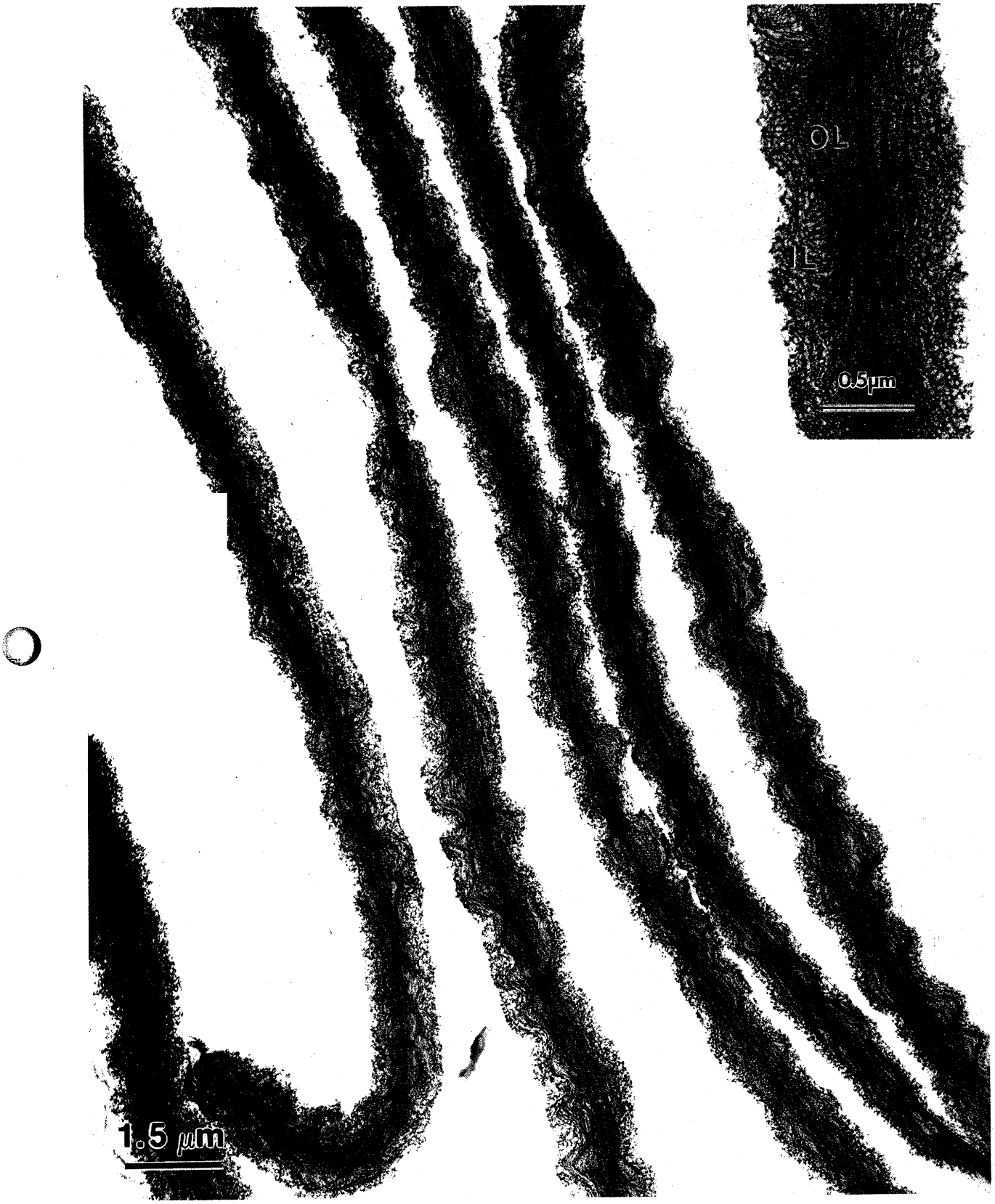

Fig. 3. Electron micrographs of purified potato tuber cell walls showing no visible membrane or organelle contamination. Insert is a much higher magnification which shows the bilayer structure (*OL* outer layer, *IL* inner layer) of the cell wall. Ten micrographs were analyzed from different areas of the purified cell wall preparation and the micrograph shown is representative

examination of the crude wall fraction immediately after cell disruption in the nitrogen bomb. The crude homogenate was centrifuged at 1,000 **g** for 5 minutes to pellet cell walls and starch bodies. Methylene blue is typically used as a contrast stain for light microscopy; however, it was difficult to resolve the presence or absence of starch bodies (micrograph not shown). India ink was used to enhance the contrast between starch grains and cell wall (Fig. 2) and after subsequent washing of the crude wall fraction, no intact cells or starch grains were apparent (Fig. 2).

Since light microscopy could not resolve the presence of either membranes or cytoplasmic fragments, purified cell walls were examined at the ultrastructural level. No membrane vesicles, organelles, or cytoplasmic fragments were observed in the purified cell wall preparation (Fig. 3). In addition, the purified cell walls had an interesting "bilayer" structure which could only be detected at the ultrastructural level. The cell walls had a loosely packed fibrillar region on the innermost surface of the wall (Fig. 3). Higher magnification showed that this fibrillar material was actually part of the cell wall (Fig. 3 insert). This layer of "vesicular" material was reported earlier for intact potato tuber cells and was discussed at length (LYSHEDE 1978 and 1979). The fibrillar layer (inner layer) was located immediately apposed to the outside surface of the plasma membrane (LYSHEDE 1978). This fibrillar layer was not unique to potato cell walls since it also has been shown in wheat and barley aleurone cell walls (BACIC and STONE 1981). This layer may contain pectin since the very early report on the ultrastructural localization of pectin showed its presence in the middle lamella as well as at the inner surface of the primary cell wall adjacent to the plasma membrane (ALBERSHEIM *et al.* 1960).

To confirm the purity of cell walls as judged by morphology, purified cell walls were monitored biochemically with markers used in the negative mode. Neither the organelle marker (CCO) nor cytoplasmic enzyme marker (MDH) were detected in the purified cell walls (Tab. 1). MDH appeared to be a good marker for a soluble enzyme since it was not detected in purified potato tuber cell walls (Tab. 1), or purified corn root cell walls (NAGAHASHI *et al.* 1985). In addition, phosphatidyl choline (PC) was monitored as a general marker for membranes. The unwashed cell walls contained 3% of the total PC, determined by the method of DITTMER and WELLS 1969, while the washed cell walls (purified) contained a barely detectable level (less than 0.1%) even when a large aliquot of cell walls was used for analysis. This very low level of PC may be due to the presence of plasmodesmata in the purified cell walls.

Table 1. *Distribution of enzymatic activity between potato tuber cell walls and cytoplasmic components.* The percent distribution was based on the total activity recovered in purified cell walls divided by this activity plus the total activity found in the cytoplasm (initial filtrate plus subsequent washings)

Enzyme activity	% cell wall	% cytoplasmic
β-galactosidase	10.2	89.8
β-glucosidase	25.4	74.6
β-NAcgalactosaminidase	41.1	58.9
β-NAcglucosaminidase	30.6	69.4
α-mannosidase	13.5	86.5
β-xylosidase	15.5	84.5
Acid phosphatase	2.0	98.0
Malate dehydrogenase	0	100
Cytochrome C oxidase	0	100

The distribution of various hydrolytic enzymes between the purified cell walls and the cytosol was also determined (Tab. 1). All hydrolytic enzymes tested (with the exception of acid phosphatase) had greater than 10% of their total activity associated with cell walls. The estimation of cell-wall associated enzyme activity was probably on the lower side since the original filtrates and combined washings were used as the "cytoplasmic source", and this combined filtrate contained small fragments of cell walls which passed through the cheesecloth. The combined cytoplasmic filtrate was placed in a beaker on ice for 15 minutes to allow starch bodies and presumably small cell wall fragments to sediment. Aliquots were then removed to estimate the cytoplasmic activity. Although the percent distribution of enzyme activity between the cell wall and the cytoplasm may not be completely accurate due to the loss of cell wall fragments during purification, these results clearly indicated that considerable particulate activity as well as soluble activity were present in potato tuber cells.

3.2. *NaCl and $CaCl_2$ Extraction of Purified Cell Walls*

Initially, purified cell walls were packed in a column and eluted with a 0–1.0 M NaCl gradient. Since considerable protein was released at approximately 0.2 M NaCl, this concentration was used for bulk extraction experiments. Proteins solubilized under these conditions were directly compared to those extracted by 0.5 M $CaCl_2$ (Fig. 4) because there has been a recent trend to use $CaCl_2$ (0.1 M to 0.5 M) for solubilizing cell wall associated proteins (COOPER *et al.* 1984, SCOTT and O'NEILL 1984).

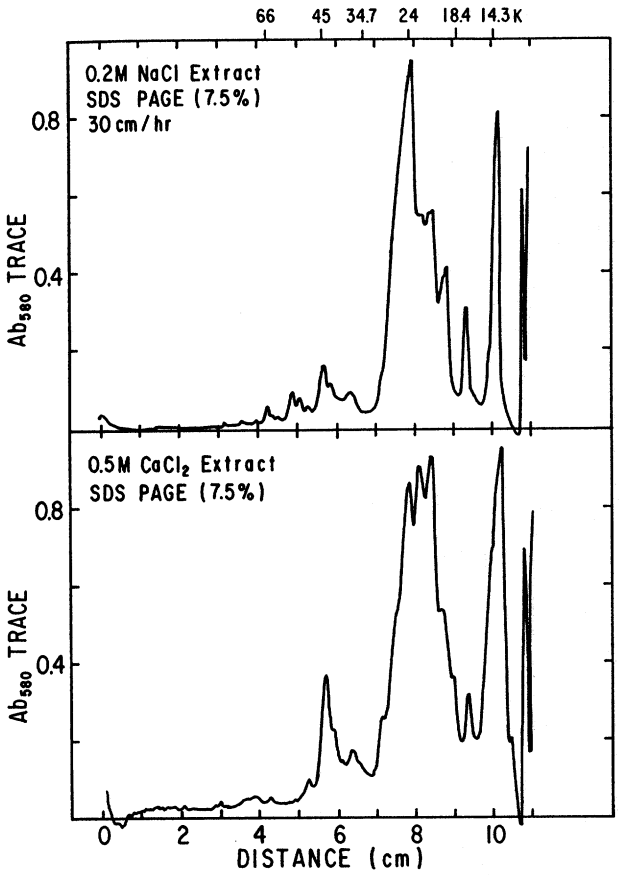

Fig. 4. SDS polyacrylamide gel (7.5%) electrophoresis of protein extracted from purified potato tuber cell walls with either 0.2 M NaCl (top) or 0.5 M $CaCl_2$ (bottom). Gels were stained with Coomassie blue and scanned at 580 nm

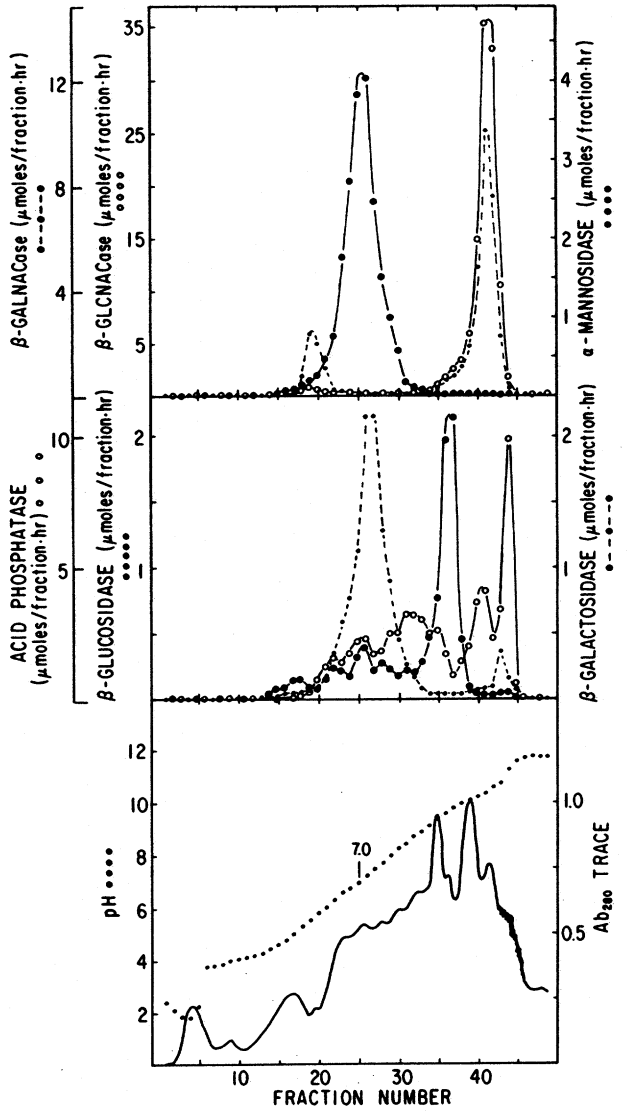

Fig. 5. Isoelectric focusing of potato tuber proteins extracted from purified cell walls. Proteins associated were extracted with 0.5 M $CaCl_2$, concentrated, and dialyzed prior to IEF. The pI's for the following enzymes were determined: β-galactosidase, 10.7, 7.3; β-glucosidase 9.7, 7.2; β-NAc-galactosaminidase, 10.5, 5.7, β-NAC-glucosaminidase, 10.5; α-mannosidase, 7.2; acid phosphatase, 11.0, 10.2, 8.7, 7.2. IEF was performed on two separate extractions and the data shown is representative with the exception of acid phosphatase activity. In the second analysis, the pI 10.2 enzyme was not detected

Although some quantitative differences in protein profile were observed between the NaCl and $CaCl_2$ (Fig. 4), we do not know whether cation specificity or the different ionic strengths of the extraction salts were mainly responsible for the observed differences. Most of the salt extracted proteins had a low molecular weight range (14 to 31 kDa) and this result was in agreement with a recent report (SCOTT and O'NEILL 1984) which used $CaCl_2$ to extract proteins from cell walls of intact carrot suspension-cultured cells.

3.3. IEF of Potato Tuber Proteins

The $CaCl_2$ extract from purified cell walls was concentrated, dialyzed against 1 mM β-mercaptoethanol and applied to an IEF column (Fig. 5). The protein distribution (A 280 nm trace) was directly compared to that of cytosolic proteins (Fig. 6). Although some acidic proteins were associated with cell walls and some basic proteins were found in the cytosol, in general, most cell wall proteins were basic in nature (pI > 7.0).

The distribution of enzyme activities was consistent with the protein distribution for both the cell wall preparation and the cytosolic preparation. With the exception of β-NAcgalactosaminidase (β-galNAcase), all of the cell wall extracted enzymes had a alkaline pI (Fig. 5) compared to the acidic pI of the cytosolic

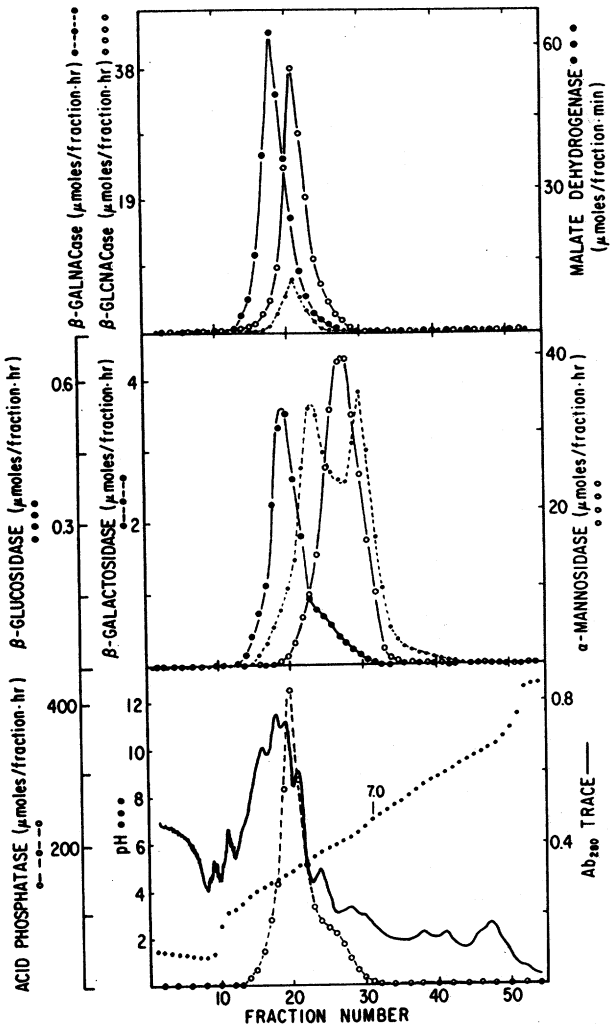

Fig. 6. Isoelectric focusing of cytosolic associated potato tuber proteins. The 100,000 g supernatant from potato tuber homogenates was fractionated with 80% ammonium sulfate and dialyzed prior to IEF. The pI's for various cytosolic enzymes were determined: β-galactosidase, 6.7, 5.7; β-glucosidase, 4.8; β-NAc-galactosaminidase 5.2; β-NAc-glucosaminidase, 5.2; α-mannosidase, 6.2; acid phosphatase, 4.9; malate dehydrogenase 4.7. This experiment was performed twice and the data shown is representative

isozymes (Fig. 6). The major peak of β-galNAcase activity was coincident with the β-NAcglucosaminidase (β-glcNAcase) activity suggesting that PNP-β-glcNAc was the preferred substrate as indicated by the much higher level of activity (Fig. 5). The second peak of β-galNAcase was unique since this enzyme did not hydrolyze PNP-glcNAc and it was the only cell wall associated enzyme with an acidic pI. β-galNAc has been reported in plant cell walls (HORI 1978, WOLD and HILLESTAD 1976) and its presence would be consistent with a potential role for β-galNAcase in cell wall metabolism.

Cell wall associated β-galactosidase activity had a major peak with a pI at 10.7 and a minor peak at 7.3. β-glucosidase also had a major peak (pI 9.7) and a minor peak (pI 7.2) and the high pI form was consistent with the only other reported value for cell wall extracted β-glucosidase activity (HÖSEL *et al.* 1978). At least four isozymes of acid phosphatase and possibly as many as seven were associated with the cell wall (Fig. 5). The pI of the most alkaline form probably was underestimated, since this peak registered in the nonlinear step area of the pH gradient. Multiple forms of acid phosphatase were expected, since a previous report showed multiple isozymes (separated by column chromatography) could be extracted from potato tuber cell walls (SUGAWARA *et al.* 1981).

The cell wall extracted α-mannosidase had a single peak of activity with a pI of 7.2. This was the highest pI reported for any α-mannosidase and it was considerably higher than soluble α-mannosidases (compare Figs. 5 and 6). Overall, the alkaline pI's reported here for cell wall associated enzymes were considerably higher than those reported for soluble enzymes (DEY and DEL CAMPILLO 1984). These results were consistent with the alkaline pI range for nonenzymatic proteins associated with cell walls (COOPER *et al.* 1984, LEACH *et al.* 1982).

Because of the remarkable contrast in isoelectric pH between cell-wall associated enzymes and the cytosolic isozymes, it could be argued that positively charged enzymes artifactually bound to the wall during the isolation procedure. This possibility was very unlikely for several reasons. First, if basic enzymes were not unique to the cell wall, they should have been detected in the soluble cytoplasm, but were not (Fig. 6). Second, the homogenization was performed at pH 7.8 which was above the pI of several cell-wall associated enzymes (Fig. 5). These enzymes would not have a strong positive charge under these conditions but they were nonetheless "bound" to the wall. Third, when cyt c (pI = 10.6) was added to the homogenization medium as an exogenous protein, less than 2% of the cyt c was bound to the cell walls during the nitrogen bomb disruption process. The poor binding was due to the high molarity (0.1 M buffer) as well as high pH (7.8) of the homogenization medium. This was demonstrated by performing cyt c binding studies with purified, salt extracted, exhaustively washed cell walls. Cyt c binding decreased dramatically when buffer concentrations greater than 20 mM were used in the binding studies. Binding studies using 15 mM buffer at various pH indicated that cyt c binds at a maximum between pH 5

Table 2. *Purified potato tuber cell walls sequentially extracted with various salts.* Each extraction step was performed for 12 hours (3 changes per salt) in the presence of 1 mM 2-mercapto ethanol at 4 °C. After each step, cell walls were washed in 1 mM 2-mercaptoethanol and cell wall aliquots were assayed directly for various hydrolytic enzymes

Enzyme	Total activity (μmoles/fraction · hour)				
	Unextracted cell walls	0.2 M NaCl extracted cell walls	0.5 M $CaCl_2$ extracted cell walls	3 M LiCl extracted cell walls	% retained by cell wall
β-galactosidase	34.87	21.29	13.06	9.58	27.5
β-glucosidase	69.94	37.33	6.39	5.33	7.9
β-NAcglucosaminidase	202.71	113.03	19.73	13.77	6.8
α-mannosidase	230.01	209.36	185.26	184.55	80.2
β-xylosidase	10.80	2.80	1.85	1.23	11.4
Acid phosphatase	383.03	337.92	144.94	105.12	27.4

to 6 and minimally at pH 8 to 9. Fourth, the large dilution used in the N_2 bomb (700 ml) would help minimize the binding of cytosolic enzymes to the isolated walls. Finally, it was previously reported that the binding of proteins to cell walls was not apparently a function of the pI of the protein (JANSEN *et al.* 1960).

3.4. Effects of Sequential Salt Extraction on the Enzyme Distribution of Purified Cell Walls

The solubilization of various hydrolytic cell wall associated enzymes was also followed during sequential salt extraction (Tab. 2). For this study, the final extraction step was with 3 M LiCl since previous workers used this salt for solubilizing cell wall proteins (VOIGHT 1985) especially α-mannosidase activity (GREVE and ORDIN 1977). To ensure that the differences observed were not due to enzyme inactivation by the various salts, the soluble extracts were combined, concentrated, dialyzed, and assayed for enzyme activity. The extracted activity plus the remaining cell wall activity were totaled and compared to the original untreated cell wall activity. In all cases, recovery was 95% or greater.

Most of the β-glcNAcase (93%) and β-glucosidase (92%) activity was solubilized with sequential salt treatment (Tab. 2). Some enzymes were not as readily solubilized since approximately 27% of the cell wall β-galactosidase and acid phosphatase activity were retained by the cell wall. Most striking was the fact that 80% of the cell wall bound α-mannosidase activity could not be solubilized. Similar results were observed for corn root all walls and apple cell walls purified as in Fig. 1. After sequential salt extraction, 85% of the corn root cell-wall associated enzyme and 76% of the apple cell wall α-mannosidase was still bound. Two previous reports (GREVE and ORDIN 1977, AHMED and LABAVITCH 1980) did not check the extracted cell walls for residual activity so it was likely they overlooked this observation.

Potato tubers have three isozymes of α-mannosidase; one that was cytosolic (pI 6.2), one that was ionically bound to the cell wall (pI 7.2) and one that appears to be covalently linked to the cell wall. Because corn root and apple cell walls also contained a tightly bound α-mannosidase, this enzyme activity can possibly be used as a marker for cell walls provided the bound form can be distinguished from the soluble forms. We attempted to inhibit selectively the cytosolic form with swainsonine (a specific inhibitor of soluble α-mannosidases); however, all three forms of the potato tuber enzyme were completely inhibited with 50 ng/ml of the compound. Since we could not distinguish the cell-wall bound α-mannosidase by selective inhibition, we are currently attempting to solubilize this enzyme with pectinases in order to determine if any of its properties are unique. We also plan to determine if cell wall enzymes such as α-mannosidase and β-glcNAcase can hydrolyze fungal cell walls and therefore play a role in host defense systems.

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